MN/CA IX AND CANCER PROGNOSIS

FIELD OF THE INVENTION

The present invention is in the general area of medical genetics and in the fields of biochemical engineering, immunochemistry and oncology. More specifically, it relates to the MN gene -- a cellular gene considered to be an oncogene, known alternatively as MN/*CA9*, *CA9*, or carbonic anhydrase 9, which gene encodes the oncoprotein now known alternatively as the MN protein, the MN/CA IX isoenzyme, MN/CA IX, carbonic anhydrase IX, CA IX or the MN/G250 protein.

More specifically, the instant invention is directed to the identification of MN antigen or MN gene expression in patient samples, which provides the basis for diagnostic/prognostic assays for cancer and for making clinical decisions on cancer treatment. Still more specifically, the instant invention concerns methods which are prognostic for patients with a preneoplastic/neoplastic disease, wherein said disease affects a tissue, which normally expresses MN protein, but wherein said tissue loses or has significantly reduced MN expression upon carcinogenesis. Gastric cancer is exemplary of such a neoplastic disease.

BACKGROUND

As indicated above, the MN gene and protein are known by a number of alternative names, which names are used herein interchangeably. The MN protein was found to bind zinc and have carbonic anhydrase (CA) activity and is now considered to be the ninth carbonic anhydrase isoenzyme – MN/CA IX or CA IX [4]. According to the carbonic anhydrase nomenclature, human CA isoenzymes are written in capital roman letters and numbers, whereas their genes are written in italic letters and arabic numbers. Alternatively, "MN" is used herein to refer either to carbonic anhydrase isoenzyme IX (CA IX) proteins/polypeptides, or carbonic anhydrase isoenzyme 9 *(CA9)* gene, nucleic acids, cDNA, mRNA etc. as indicated by the context.

The MN protein has also been identified with the G250 antigen. Uemura et al. [35] states: "Sequence analysis and database searching revealed that

G250 antigen is identical to MN, a human tumor-associated antigen identified in cervical carcinoma (Pastorek et al., 1994)."

Zavada et al., International Publication Number WO 93/18152 (published Sep. 16, 1993) and U.S. Pat. No. 5,387,676 (issued Feb. 7, 1995), describe the discovery of the MN gene and protein. The MN gene was found to be present in the chromosomal DNA of all vertebrates tested, and its expression to be strongly correlated with tumorigenicity. In general, oncogenesis may be signified by the abnormal expression of CA IX protein. For example, oncogenesis may be signified: (1) when CA IX protein is present in a tissue which normally does not express CA IX protein to any significant degree; (2) when CA IX protein is absent from a tissue that normally expresses it; (3) when *CA9* gene expression is at a significantly increased level, or at a significantly reduced level from that normally expressed in a tissue; or (4) when CA IX protein is expressed in an abnormal location within a cell.

The MN protein is now considered to be the first tumor-associated carbonic anhydrase isoenzyme that has been described. The carbonic anhydrase family (CA) includes eleven catalytically active zinc metalloenzymes involved in the reversible hydration-dehydration of carbon dioxide: $CO_2 + H_20 \Leftrightarrow HCO_3^- + H^+$. CAs are widely distributed in different living organisms. The CAs participate in a variety of physiological and biological processes and show remarkable diversity in tissue distribution, subcellular localization, and biological functions [1, 2, 27]. Carbonic anhydrase IX, CA IX, is one of the most recently identified isoenzymes [3, 4]. Because of the CA IX overexpression in transformed cell lines and in several human malignancies, it has been recognized as a tumor-associated antigen and linked to the development of human cancers [5-7].

CA IX is a glycosylated transmembrane CA isoform with a unique N-terminal proteoglycan-like extension [4]. Through transfection studies it has been demonstrated that CA IX can induce the transformation of 3T3 cells [4]. Recent studies have revealed that CA IX not only participates in cell adhesion, but also can be induced in hypoxia via the HIF-1 protein binding to the hypoxia-responsive element of the MN promoter [8, 9]. The transcription of the MN gene is negatively regulated by the von Hippel-Lindau tumor suppressor gene in renal cell carcinoma cells [28]. The protein product of the von Hippel-Lindau tumor suppressor gene

interacts with the ubiquitin ligase complex that is responsible for targeting HIF-1α for oxygen-dependent proteolysis [29, 30]. Thus, low levels of oxygen lead to stabilization of HIF-1α, which in turn leads to the increased expression of MN [9]. Areas of high expression of MN in cancers are linked to tumor hypoxia as reported in many cancers and incubation of tumor cells under hypoxic conditions leads to the induction of MN expression [9-14] .

Many studies have confirmed the diagnostic/prognostic utility of MN, using the MN-specific monoclonal antibody (MAb) M75 in diagnosing/prognosing precancerous and cancerous cervical lesions [6, 37, 38, 39, 55]. Immuno-histochemical studies with the M75 MAb of cervical carcinomas and a PCR-based (RT-PCR) survey of renal cell carcinomas have identified MN expression as closely associated with those cancers and indicates that MN has utility as a tumor biomarker [6, 36, 38]. In various cancers (notably uterine cervical, ovarian, endometrial, renal, bladder, breast, colorectal, lung, esophageal, head and neck and prostate cancers, among others), CA IX expression is increased and has been correlated with the microvessel density and the levels of hypoxia in some tumors [10, 11].

In tissues that normally do not express MN protein, CA IX positivity is considered to be diagnostic for preneoplastic/neoplastic diseases, such as, lung, breast and cervical cancers [12-14]. However, among those cancerous tissues, higher MN expression often indicates a better prognosis. Previous studies have reported that there is an inverse correlation between CA IX expression and stage and grade in some tumors, including clear cell RCC [40], cervical carcinoma [39], colorectal tumors [7], and esophageal cancer [52]. Of these studies, the three that were non-RCC-related found that low expression of CA IX correlated with poor prognostic factors, such as lymph node metastases and depth of invasion. Bretheau et al. 1995 [41] reported the poor prognosis of RCC patients with high grade and stage tumors, which according to Uemura et al. [40] would be expected to express CA IX at lower levels. Bui et al. [42; International Publication No. WO 03/089659] reported that "low" CA IX ($\leq$85%) staining was an independent poor prognostic factor for survival for patients with metastatic RCC.

Very few normal tissues have been found to express MN protein to any significant degree; those MN-expressing normal tissues include the human gastric mucosa and gallbladder epithelium, and some other normal tissues of the alimentary

tract [45, 15, 16]. Immunohistochemical analysis of the normal large intestine revealed moderate CA IX staining in the proximal colon, with the reaction becoming weaker distally. The staining was confined to the basolateral surfaces of the cryptal epithelial cells, the area of greatest proliferative capacity. As CA IX is much more abundant in the proliferating cryptal epithelium than in the upper part of the mucosa, it may play a role in control of the proliferation and differentiation of intestinal epithelial cells. Cell proliferation increases abnormally in premalignant and malignant lesions of the colorectal epithelium, and therefore, is considered an indicator of colorectal tumor progression. [43, 44]. Interestingly, *CA9* deficient mice develop gastric hyperplasia which is associated with increased proliferation [17], raising the question, whether the putative pathophysiological role of CA IX in gastric cancer development and progression is different from the one observed in cancers of non-gastric origin.

Gastric cancer is the second most common cause of cancer-related deaths worldwide [22, 23]. Despite its decreasing incidence it remains a great challenge for clinicians and oncologists. In recent years various groups have analysed the genetic and molecular changes leading to gastric cancer. Those changes include, among others, the overexpression of oncogenes, such as growth factor receptors *K-sam* and *c-met*, the loss of certain tumor suppressor genes, such as *APC* and *p53*, as well as alteration of adhesion molecules, including E-cadherin and the catenins [22-26]. Recently, the group of carbonic anhydrases and especially CA IX have received increasing attention [2]. However, studies to date investigating CA IX expression in gastric mucosa, which normally overexpresses CA IX, have provided only diagnostic analysis, associating the presence of gastric cancer with diminished levels of CA IX or loss of CA IX.

Disclosed herein is a surprising finding that has led to novel and inventive prognostic methods for gastric cancer and related cancers, that are diagnosed by the loss or reduction of the CA IX expression, that is abundant in corresponding normal tissue. Surprisingly, among such cancers, it was found that CA IX expression that is higher than the absent or significantly reduced levels of CA IX expression considered diagnostic for gastric and related cancers, indicates a poorer prognosis for patients that have been diagnosed with such cancers, particularly when such higher CA IX expression is found at the invasion fronts of

such cancers. Disclosed herein is the surprising finding that in gastric cancer and related cancers, a higher expression of CA IX indicates a poorer prognosis for afflicted patients, particularly when expressed at the invasion front of the cancer.

SUMMARY OF THE INVENTION

The present invention relates to prognostic methods comprising quantitating levels of MN/*CA9* gene expression products in patients afflicted with preneoplastic/neoplastic diseases of tissues, wherein the normal tissue associated with such a preneoplastic/neoplastic disease expresses CA IX, but loses CA IX expression upon carcinogenesis. Quantitating such levels of MN/CA9 gene expression product is useful in determining prognosis of the patient. Such tissue is preferably selected from the group consisting of gastric mucosa, gallbladder, biliary ducts, ductal cells of duodenal glands, testis including ductular efferens and rete testis, ovary including surface coelomic epithelium and rete ovarii, basal cells of hair follicles, and central nervous system choroid plexus. More preferably, said tissue is gastric mucosa, gallbladder, biliary ducts or ductal cells of duodenal glands; still more preferably, said tissue is gastric mucosa, gall bladder or biliary ducts; further preferably said tissue is gastric mucosa.

A first prognostic method comprises quantitating the level of a MN/*CA9* gene expression product in a tissue sample taken from a patient, and comparing that level with levels of MN/*CA9* gene expression products in comparable tissue samples from patients afflicted with the same disease. A second prognostic method comprises analyzing the tissue sample from the invasion front of said preneoplastic/neoplastic disease in said patient and comparing the MN/*CA9* gene expression level to the levels normally found in said tissue. In addition to predicting clinical outcome, the methods of the present invention also identify high-risk patients in need of adjuvant therapy, and/or CA IX-targeted therapies, among other courses of treatment.

In one aspect, the invention concerns methods which are prognostic for a preneoplastic/neoplastic disease afflicting a subject vertebrate, preferably a mammal, wherein said disease affects a tissue, which tissue normally expresses MN/CA IX protein, but loses or has significantly reduced MN/CA IX expression upon carcinogenesis, said method comprising:

(a) detecting MN/*CA9* gene expression product in a sample comprising preneoplastic/neoplastic tissue taken from said vertebrate,

(b) quantitating the level of said MN/*CA9* gene expression product in said sample,

(c) comparing the level of MN/*CA9* gene expression product of step (b) to the average level of MN/*CA9* gene expression product in comparable samples taken from vertebrates afflicted by the same preneoplastic/neoplastic disease as the subject vertebrate, and

(d) determining that said subject vertebrate has a poorer prognosis if the level of MN/*CA9* gene expression product of step (b) is higher than the average level of MN/*CA9* gene expression product in said comparable samples;

wherein said MN/CA IX protein is encoded by a nucleotide sequence selected from the group consisting of:

(1) SEQ ID NO: 1's coding region;

(2) nucleotide sequences that hybridize under stringent hybridization conditions of 50% formamide at 42 degree C. to complement of SEQ ID NO: 1's coding region; and

(3) nucleotide sequences that differ from SEQ ID NO: 1's coding region or from the nucleotide sequences of (2) in codon sequence due to the degeneracy of the genetic code.

Preferred assays to be used according to the methods of the invention to detect said MN/*CA9* gene expression product in detecting step (a) are those wherein said MN/*CA9* gene expression product comprises an MN/CA IX protein or MN/CA IX polypeptide, and said assays are selected from the group consisting of Western blots, enzyme-inked immunosorbent assays, radioimmunoassays, competition immunoassays, dual antibody sandwich assays, immunohistochemical staining assays, agglutination assays, fluorescent immunoassays, and cytofluorometry. More preferably, said MN/*CA9* gene expression product detecting step (a) is by immunohistochemical staining, and said quantitating step (b) comprises determining the percentage of immunoreactive cells and/or the intensity of immunostaining of immunoreactive cells, preferably comprising the addition or multiplication of said percentage of immunoreactive cells and said intensity of immunostaining of immunoreactive cells. Still more preferably, said detecting step (a)

comprises the use of the MN-specific M75 monoclonal antibody secreted by the hybridoma VU-M75 which has Accession No. ATCC HB 11128.

In a preferred embodiment of the invention, the MN/*CA9* gene expression product is CA IX antigen, and the CA IX antigen is quantitated in preneoplastic/neoplastic vertebrate samples, preferably mammalian samples, more preferably human samples. Such preneoplastic/neoplastic samples can be tissue specimens, tissue extracts, cells, cell lysates and cell extracts, among other samples. Such tissue specimens can be variously maintained, for example, they can be fresh, frozen, or formalin-, alcohol- or acetone- or otherwise fixed and/or paraffin-embedded and deparaffinized. Preferred tissue samples are formalin-fixed, paraffin-embedded tissue samples or frozen tissue samples. In a preferred embodiment, the disease is gastric cancer, and the sample is taken from the invasion front of the gastric cancer. Preferably, said sample is taken from the invasion front of said preneoplastic/neoplastic disease, preferably a neoplastic disease, and said comparable samples used in comparing step (c) are analogous invasion front samples.

An exemplary and preferred method which is prognostic for a preneoplastic/neoplastic disease affecting a subject vertebrate, wherein said disease affects a tissue, which tissue normally expresses MN/CA IX protein, but loses or has significantly reduced MN/CA IX expression upon carcinogenesis, comprises:

(a) detecting MN/*CA9* gene expression product in a sample comprising preneoplastic/neoplastic tissue taken from said vertebrate, said detecting comprising immunohistochemical staining with MN/CA IX-specific antibody to detect MN/CA IX protein in the sample;

(b) quantitating the level of said MN/*CA9* gene expression product in said sample, comprising:

(b1) determining the percentage of immunoreactive cells, wherein the percentage of immunoreactive cells is assigned

a value of 0 if no immunoreactive cells,

a value of 1 if less than 10% immunoreactive cells,

a value of 2 if between 11% and 50% immunoreactive cells, or

a value of 3 if more than 50% immunoreactive cells;

(b2) determining the intensity of immunostaining of the immunoreactive cells, wherein the intensity of MN/CA IX immunostaining is assigned

a value of 0 for staining equal to a negative control,

a value of 1 for weak staining,

a value of 2 for moderate staining, or

a value of 3 for strong staining; and

(b3) adding the value for the percentage of immunoreactive cells found in step (b1) and the value for the intensity of immunostaining found in step (b2) to obtain the immunoreactivity score;

(c) comparing the immunoreactivity score of the subject vertebrate found in step (b) to the average immunoreactivity score in comparable samples taken from vertebrates afflicted by the same preneoplastic/neoplastic disease as the subject vertebrate, comprising determining the immunoreactivity scores of said comparable samples analogously to the determination of the immunoreactivity score of the sample from the subject vertebrate in steps (b1) to (b3), and averaging said immunoreactivity scores from said comparable samples; and

(d) determining that said subject vertebrate has a poorer prognosis if said immunoreactivity score of the sample determined in steps (b1) to (b3) is above the average immunoreactivity score of said comparable samples found in step (c);

wherein said MN/CA IX protein is encoded by a nucleotide sequence selected from the group consisting of:

(2) SEQ ID NO: 1's coding region;

(2) nucleotide sequences that hybridize under stringent hybridization conditions of 50% formamide at 42 degree C. to complement of SEQ ID NO: 1's coding region; and

(3) nucleotide sequences that differ from SEQ ID NO: 1's coding region or from the nucleotide sequences of (2) in codon sequence due to the degeneracy of the genetic code.

In an alternative preferred embodiment, preferred assays to be used according to the methods of the invention in said MN/*CA9* gene expression product detecting step (a) are nucleic acid-based assays, wherein said MN/*CA9* gene expression product comprises a mRNA encoding an MN/CA IX protein or MN/CA IX polypeptide, or a cDNA complementary to mRNA encoding an MN/CA IX protein or

MN/CA IX polypeptide. Preferably, said detecting step (a) is by PCR, RT-PCR, real-time PCR, or by quantitative real-time RT-PCR.

Preferably, the preneoplastic/neoplastic disease to be tested according to the prognostic methods of the invention for MN/*CA9* gene expression product, is a disease which affects a tissue wherein 40% or more of the cells of said tissue, when unaffected by said preneoplastic/neoplastic disease, express MN/CA IX protein. Preferably said preneoplastic/neoplastic disease afflicting the subject vertebrate is selected from the group consisting of preneoplastic/neoplastic diseases of gastric mucosa, gallbladder, biliary ducts, ductal cells of duodenal glands, testis including ductular efferens and rete testis, ovary including surface coelomic epithelium and rete ovarii, basal cells of hair follicles, and central nervous systems choroid plexus. More preferably, said preneoplastic/neoplastic disease is selected from the group consisting of preneoplastic/neoplastic diseases of gastric mucosa, gallbladder, bilary ducts, and ductal cells of duodenal glands. Preferably said vertebrate is a mammal, more preferably human. Still more preferably, the vertebrate is a human patient, and said preneoplastic/neoplastic disease is selected from the group consisting of neoplastic diseases of gastric mucosa, gallbladder, biliary ducts and ductal cells of duodenal glands. Most preferably, said neoplastic disease is gastric cancer, and said sample is taken from the invasion front of said gastric cancer. Preferably, said neoplastic disease is a tumor, and said sample is taken from said tumor and/or from a metastatic lesion derived from said tumor.

Preferred prognostic methods according to the invention are those wherein a poorer prognosis is measured in terms of shortened survival, increased risk of recurrence of said preneoplastic/neoplastic disease, or in diminished or refractory response to treatment. Further preferred methods are those wherein said disease is neoplastic and comprises a tumor, or a tumor and one or more metastatic lesions derived from the tumor, and wherein a poorer prognosis is measured in terms of shortened survival, increased risk of recurrence of said neoplastic disease, or diminished or refractory response to treatment, following treatment and/or surgical removal of the tumor, or the tumor and said one or more metastatic lesions. Preferably, said prognostic method is used as an aid in the selection of treatment for said preneoplastic/neoplastic disease afflicting said vertebrate. Exemplary treatments include chemotherapy, radiation, and/or surgery.

In another aspect, this invention concerns methods which are prognostic for a preneoplastic/neoplastic disease afflicting a subject vertebrate, wherein said disease affects a tissue in which 40% or more of the cells normally express MN/CA IX protein, but said tissue loses or expresses MN/CA IX at a significantly reduced level upon carcinogenesis, comprising:

(a) taking a tissue sample from the invasion front of said preneoplastic/neoplastic disease;

(b) detecting in said invasion front sample whether MN/*CA9* gene expression product is absent or at a significantly reduced level from the level that said MN/*CA9* gene expression product is normally expressed in said tissue, when said tissue is unaffected by said disease; and

(c) concluding that if said MN/*CA9* gene expression product is neither absent nor at such a significantly reduced level in said invasion front sample, that the subject vertebrate has a poorer prognosis than if said MN/*CA9* gene expression product were absent or at a such a significantly reduced level in said invasion front sample.

Aspects of the instant invention disclosed herein are described in more detail below.

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Abbreviations

The following abbreviations are used herein:

aa	-	amino acid
ATCC	-	American Type Culture Collection

	AZA	-	azadeoxycytidine
	bp	-	base pairs
	BRL	-	Bethesda Research Laboratories
	CA	-	carbonic anhydrase
5	°C	-	degrees centigrade
	CDR	-	complementarity determining region
	DMEM	-	Dulbecco modified Eagle medium
	DMSO	-	dimethyl sulfoxide
	ds	-	double-stranded
10	DTT	-	dithiothreitol
	EDTA	-	ethylenediaminetetraacetate
	FCS	-	fetal calf serum
	FITC	-	fluorescein isothiocyanate
	HRP	-	horseradish peroxidase
15	IC	-	intracellular
	IRS	-	immunoreactivity score
	kb	-	kilobase
	kbp	-	kilobase pairs
	kd or kDa	-	kilodaltons
20	LTR	-	long terminal repeat
	M	-	molar
	MAb	-	monoclonal antibody
	ME	-	mercaptoethanol
	min.	-	minute(s)
25	mg	-	milligram
	ml	-	milliliter
	mM	-	millimolar
	MMLV	-	Moloney murine leukemia virus
	mmol	-	millimole
30	N	-	non-neoplastic gastric mucosa
	ng	-	nanogram
	nm	-	nanometer
	nM	-	nanomolar

nt	-	nucleotide
ORF	-	open reading frame
PCR	-	polymerase chain reaction
PG	-	proteoglycan
pI	-	isoelectric point
pmol	-	picamolar
RCC	-	renal cell carcinoma
RNP	-	RNase protection assay
RT-PCR	-	reverse transcription polymerase chain reaction
SDS	-	sodium dodecyl sulfate
SP	-	signal peptide
SSP	-	standard saline phosphate ethylenediaminetetraacetic acid
T	-	gastric tumor
TM	-	transmembrane
Tris	-	tris (hydroxymethyl) aminomethane
U	-	units
μg	-	microgram
μl	-	microliter
μM	-	micromolar

Cell Lines

AGS - human gastric cancer cell line; gastric adenocarcinoma [American Type Culture Collection (ATCC), Rockville, MD]

HeLa cells - human cervical cancer cell line; epithelial adenocarcinoma [American Type Culture Collection (ATCC), Rockville, MD]

MKN45 - human gastric cancer cell line; poorly differentiated adenocarcinoma [Riken Cell Bank, Tsukuba, Japan]

MKN28 - human gastric cancer cell line; moderately differentiated tubular adenocarcinoma [Riken Cell Bank, Tsukuba, Japan]

N87 - human gastric cancer cell line; gastric carcinoma derived in 1976 by A. Gazdar from liver metastatic site [American Type Culture Collection (ATCC), Rockville, MD]

Nucleotide and Amino Acid Sequence Symbols

The following symbols are used to represent nucleotides herein:

Base Symbol	Meaning
A	adenine
C	cytosine
G	guanine
T	thymine
U	uracil
I	inosine
M	A or C
R	A or G
W	A or T/U
S	C or G
Y	C or T/U
K	G or T/U
V	A or C or G
H	A or C or T/U
D	A or G or T/U
B	C or G or T/U
N/X	A or C or G or T/U

There are twenty main amino acids, each of which is specified by a different arrangement of three adjacent nucleotides (triplet code or codon), and which are linked together in a specific order to form a characteristic protein. A three-letter or one-letter convention is used herein to identify said amino acids, as, for example, in Figure 1 as follows:

Amino acid name	3 Ltr. Abbrev.	1 Ltr. Abbrev.
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Unknown or other		X

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A-C provides the nucleotide sequence for MN/CA IX full-length cDNA [SEQ ID NO: 1]. Figure 1 A-C also sets forth the predicted amino acid sequence encoded by the cDNA [SEQ ID NO: 2.].

Figure 2A-F provides a 10,898 bp complete genomic sequence of MN/*CA9* [SEQ ID NO: 3]. The base count is as follows: 2654 A; 2739 C; 2645 G; and 2859 T. The 11 exons are in general shown in capital letters, but exon 1 is considered to begin at position 3507 as determined by RNase protection assay.

Figure 3 provides an exon-intron map of the human MN/*CA9* gene. The positions and sizes of the exons (numbered, cross-hatched boxes), Alu repeat elements (open boxes) and an LTR-related sequence (first unnumbered stippled box) are adjusted to the indicated scale. The exons corresponding to individual MN/CA IX protein domains are enclosed in dashed frames designated PG (proteoglycan-like domain), CA (carbonic anhydrase domain), TM (transmembrane anchor) and IC (intracytoplasmic tail). Below the map, the alignment of amino acid sequences illustrates the extent of homology between the MN/CA IX protein PG region (aa 53-111) [SEQ ID NO: 4] and the human aggrecan (aa 781-839) [SEQ ID NO: 5].

Figure 4 (discussed in Example 2) shows results from Western blot analysis and real-time PCR of CA IX protein and *CA9* mRNA in gastric cancer. (A) Western blot analysis revealed reduced CA IX protein levels in gastric cancer (T) compared with the non-neoplastic gastric mucosa (N). CA IX was identified as a protein with 54 and 58 kDa. β-actin protein levels were assessed for standardization of protein levels. No CA IX protein was detected in AGS cells, whereas low levels were found in N87 and MKN28 cells. Hela cells served as a control. (B) Quantitative analysis of *CA9* mRNA and CA IX protein levels in gastric tumors (T) as assessed by Western blot analysis and real-time PCR compared to the matched non-neoplastic gastric mucosa (N). In 5 cases protein and mRNA levels were assessed in both the cancerous and non-cancerous tissues and exhibited decreased levels in the cancerous part in all cases.

Figure 5 (discussed in Example 3) provides survival analysis of patients with gastric cancer expressing low or high levels of the CA IX protein. Using an immunoreactivity score as outlined in Materials and Methods, a group of patients with a IRS ≤ 3 (CA IX-) and a second group with a IRS > 3 (CA IX+) were identified. Survival was significantly shorter in patients with increased CA IX expression (score > 3; CA IX+) ($p=0.0281$).

Figure 6 (discussed in Example 4) provides in vitro analysis of CA IX overexpression in AGS gastric cancer cells. (A) Cellular invasion of AGS cells transfected with *CA9* cDNA (CA9), incubated with the Transfectam reagent without DNA (control A) or transfected with the empty pCMVβ vector (control B) was evaluated in 24-well Transwell chambers (Costar, Bodenheim, Germany) as

described above. The differences between AGS cells transfected with the empty pCMVβ vector and the *CA9* transfected cells, as well as the cells without DNA transfer and the *CA9* transfected cells were statistically significant (two-tailed, unpaired t test; mean ± SD). Bars, mean ± SD. (B) Induction of cell proliferation by *CA9* transfection in AGS cancer cells. Transfection of AGS cells with *CA9* cDNA led to a significant induction of cell proliferation compared to cells without DNA transfection (control A) or transfected with an empty pCMVβ vector (control B) (two-tailed, unpaired t test; mean ± SD). Bars, mean ± SD.

Figure 7 (discussed in Example 5) shows analysis of methylation effects on CA IX expression in gastric cancer cell lines. (A) *CA9* mRNA levels in gastric cancer cell lines were assessed with and without incubation with 5'-azadeoxycytidine (AZA). Basal mRNA expression (white columns) was standardized in all cells and the relative changes after incubation with 5'-azadeoxycytidine (grey columns) was assessed by realtime PCR. While no significant change was observed for MKN28 cells, the other cells, i.e. AGS, MKN45 and N87 cells, exhibited a more than 5-fold increase in *CA9* mRNA levels following treatment with 5'-azadeoxycytidine (AZA). (B) Cellular invasion of AGS cells treated with or without DMSO or 5'-azadeoxycytidine was evaluated in 24-well Transwell (8 µm pore size) chambers (Costar, Bodenheim, Germany). Invading cells were harvested from the lower side of the filters by using trypsin/EDTA. Cell number was quantified in a Coulter Counter ZII (Coulter Immunotech, Marseille, France). The differences between 5'-azadeoxycytidine (AZA) and untreated AGS cells (AGS, DMSO) was statistically significant (two-tailed, unpaired t test; mean ± SD). Bars, mean ± SD.

DETAILED DESCRIPTION

The novel methods of the present invention demonstrate that the gene expression products of the cancer-related CA9 gene are associated with survival of a vertebrate afflicted with a preneoplastic/neoplastic disease, wherein said disease affects a tissue which normally expresses MN/CA IX protein, but loses or has significantly reduced CA IX expression upon carcinogenesis. Exemplary of such preneoplastic/neoplastic diseases are preneoplastic/neoplastic diseases of gastric mucosa, gallbladder, biliary ducts, ductal cells of duodenal glands, testis including ductular efferens and rete testis, ovary including surface coelomic epithelium and

rete ovarii, basal cells of hair follicles, or central nervous system choroid plexus. In particular, the levels of *CA9* gene expression products can be used to predict clinical outcome and to identify high risk patients in need of adjuvant therapies.

The invention provides methods for prognosis of diseases associated with tissues that normally express CA IX protein, preferably a preneoplastic/neoplastic disease of gastric mucosa, gallbladder, biliary ducts, and ductal cells of duodenal glands. The methods include quantifying MN/CA9 gene expression product, if any, present in a sample taken from a patient diagnosed with such a preneoplastic/neoplastic disease; the MN/CA9 gene expression product can be CA IX protein, CA IX polypeptide, mRNA encoding a CA IX protein or polypeptide, a cDNA corresponding to an mRNA encoding a CA IX protein or polypeptide, or the like. The quantified MN/CA9 gene expression product levels are compared with the average levels in comparable samples taken from comparable patients, and correlated with a better or worse prognosis for the patient. Said CA9 gene expression product is preferably a CA IX protein or CA IX polypeptide quantitated in a sample taken from the patient.

The use of gene expression products of oncogenes as prognostic indicators for preneoplastic/neoplastic diseases is considered conventional by those of skill in the art. However, the application of such approaches to a preneoplastic/neoplastic disease, wherein said disease affects a tissue which normally expresses CA IX protein, but has significantly reduced CA IX expression upon carcinogenesis, is new. In contrast to the methods of prognosis for many other CA IX-associated preneoplastic/neoplastic diseases that are not the subject of the present invention, which affect tissues which normally do not express CA IX protein, (i.e., most tissue types), the methods of the present invention indicate a poorer prognosis when CA IX gene expression product is expressed at a higher level than average when compared to CA9 gene expression product levels in comparable affected tissues.

Preneoplastic/neoplastic Tissues

Preferably, said preneoplastic/neoplastic tissue is one in which 40% or more of the cells of said tissue express CA IX protein, when unaffected by preneoplastic/neoplastic disease. Exemplary normal human tissues expressing CA

IX protein at such a level, as identified, for example, by immunohistochemical staining using the monoclonal antibody M75 and exemplary matched neoplastic tissues, have previously been described in detail [45, 49]. In the gastrointestinal tract, diffuse CA IX immunoreactivity has been observed in the gastric mucosa, ductal cells of duodenal glands, and crypt cells of the duodenum, jejunum, and to a lesser degree, in the terminal ileum and appendix. High levels of CA IX expression have been consistently observed in the basal cells in and near the infundibulum and medulla of the hair follicle, mesothelial cells, and coelomic epithelium of the body cavities. In the visceral organs, high levels of CA IX expression in the epithelium have been identified but limited to rete ovarii, rete testis, ductular efferens, bile ducts, and gallbladder. In the peripheral and central nervous systems, CA IX expression is limited to the ventricular lining cells and the choroid plexus.

According to the methods of the invention, the preneoplastic/neoplastic tissue that is the subject of the invention is one that not only normally expresses CA IX protein, but also loses or has significantly reduced CA IX protein expression upon carcinogenesis, such as stomach and gallbladder tissues [16, 45, 54].

As used herein, "cancerous" and "neoplastic" have equivalent meanings, and "precancerous" and "preneoplastic" have equivalent meanings.

Intestinal metaplasia is defined to be "the transformation of mucosa, particularly in the stomach, into glandular mucosa resembling that of intestines, although usually lacking villi." [Stedman's Medical Dictionary, 26th Edition (Williams & Wilkins; Baltimore, MD, USA; 1995).].

The large intestine is defined as "the portion of the digestive tube extending from the ileocecal valve to the anus; it comprises the cecum, colon, rectum, and anal canal. SYN intestinum crassum." [Id.]

The colon is "[t]he division of the large intestine extending from the cecum to the rectum." [Id.]

Duodenal glands are "small, branched, coiled tubular glands that occur mostly in the submucosa of the first third of the duodenum; they secrete an alkaline mucoid substance that serves to neutralize gastric juice. SYN glandulae duodenales . . . , Brunner's g.'s, Wepfer's g.'s." [Id.]

In a preferred embodiment of the invention, the MN/*CA9* gene expression product is CA IX antigen, and the CA IX antigen is quantitated in

preneoplastic/neoplastic vertebrate samples, preferably mammalian samples, more preferably human samples. Such preneoplastic/neoplastic samples can be tissue specimens, tissue extracts, cells, cell lysates and cell extracts, among other samples. Preferred tissue samples are formalin-fixed, paraffin-embedded tissue samples or frozen tissue samples. In a preferred embodiment, the disease is gastric cancer, and the sample is taken from the invasion front of the gastric cancer.

It can be appreciated by those of skill in the art that various other preneoplastic/neoplastic samples can be used to quantify the CA IX gene expression products. For example, in the case of a patient afflicted with a neoplastic disease, wherein the disease is a tumor, the sample may be taken from the tumor or from a metastatic lesion derived from the tumor.

It can further be appreciated that alternate methods, in addition to those disclosed herein, can be used to quantify the *CA9* gene expression products. In preferred embodiments, the gene expression product is CA IX antigen which is detected and quantified by immunohistochemical staining (e.g., using tissue arrays or the like). Preferred tissue specimens to assay by immunohistochemical staining, for example, include cell smears, histological sections from biopsied tissues or organs, and imprint preparations among other tissue samples. An exemplary immunohistochemical staining protocol is described below in the Materials and Methods section. Such tissue specimens can be variously maintained, for example, they can be fresh, frozen, or formalin-, alcohol- or acetone- or otherwise fixed and/or paraffin-embedded and deparaffinized. Biopsied tissue samples can be, for example, those samples removed by aspiration, bite, brush, cone, chorionic villus, endoscopic, excisional, incisional, needle, percutaneous punch, and surface biopsies, among other biopsy techniques.

Assays

Assays using MN proteins/polypeptides and/or MN nucleic acids, as the methods described herein, may be both diagnostic and/or prognostic, i.e., diagnostic/prognostic. The term "diagnostic/prognostic" is herein defined to encompass the following processes either individually or cumulatively depending upon the clinical context: determining the presence of disease, determining the nature of a disease, distinguishing one disease from another, forecasting as to the

probable outcome of a disease state, determining the prospect as to recovery from a disease as indicated by the nature and symptoms of a case, monitoring the disease status of a patient, monitoring a patient for recurrence of disease, and/or determining the preferred therapeutic regimen for a patient. The diagnostic/prognostic methods of this invention are useful, for example, for screening populations for the presence of neoplastic or pre-neoplastic disease, determining the risk of developing neoplastic disease, diagnosing the presence of neoplastic and/or pre-neoplastic disease, monitoring the disease status of patients with neoplastic disease, and/or determining the prognosis for the course of neoplastic disease.

The screening methods of the instant invention are useful for screening a variety of preneoplastic/neoplastic diseases as indicated herein. It can be envisioned that at the same time that a disease, which is the subject of the prognostic methods of the instant invention, is first diagnosed, as for example, gastric cancer, that the level of MN gene expression product could also provide prognostic information. For example, a gastric cancer could be simultaneously diagnosed and prognosed; reduced or absent MN gene expression product in the bulk of a gastric tissue sample would be diagnostic, whereas the presence of significant MN gene expression product at the invasion front would be prognostic. The normal level of MN expression in non-neoplastic epithelium adjacent to such a gastric cancer would be maintained.

The assays of this invention can also be used to confirm the absence or removal of all tumor tissue following surgery, cancer chemotherapy and/or radiation therapy. It can further be used to monitor cancer chemotherapy and tumor reappearance.

Many formats can be adapted for use with the methods of the present invention. The detection and quantitation of CA IX protein or *CA9* polypeptide can be performed, for example, by Western blots, enzyme-linked immunosorbent assays, radioimmunoassays, competition immunoassays, dual antibody sandwich assays, immunohistochemical staining assays, agglutination assays, fluorescent immunoassays, cytofluorometry, immunoelectron and scanning microscopy using immunogold, among other assays commonly known in the art. The quantitation of *CA9* gene expression products in such assays can be adapted by conventional methods known in the art; for example, if the detection method is by

immunohistochemical staining, the quantitation of CA IX protein or CA IX polypeptide can be performed by determining the percentage of immunoreactive cells and/or the intensity of immunostaining of immunoreactive cells, and can additionally comprise addition or multiplication of these values, or other mathematical calculations using these values.

It is also apparent to one skilled in the art of immunoassays that CA IX proteins or polypeptides can be used to detect and quantitate CA IX antigen in body tissues and/or cells of patients. In one such embodiment, an immunometric assay may be used in which a labelled antibody made to CA IX protein is used. In such an assay, the amount of labelled antibody which complexes with the antigen-bound antibody is directly proportional to the amount of CA IX antigen in the sample.

Exemplary Immunohistochemical Assays

The distribution and expression pattern of MN/CA IX was investigated by immunohistochemistry, described in Example 1. Tissue sections were stained with the MN/CA IX-specific monoclonal antibody M75. Exemplary immunohistochemical staining results show MN/CA IX in the non-neoplastic gastric mucosa, intestinal metaplasia and significantly less often in gastric cancer. A lack of MN/CA IX immunostaining was found in gastric cancer of a moderately (G2) differentiated intestinal type and in a diffuse type of gastric cancer, whereas the neighboring non-neoplastic epithelial cells showed strong immunostaining. Occasionally the immunostaining was found to be heterogeneous. For example, a poorly differentiated (G3) intestinal type gastric cancer was found with no immunostaining of the tumor cells in the gastric mucosa, but with intense staining in a subset of the tumor cells infiltrating the muscularis propria.

Immunohistochemical analysis, described in Example 3, revealed a further important observation. After applying an immunoreactivity score, two groups of CA IX expression patterns were identified in gastric cancer. Cancers expressing abundant CA IX exhibited a shorter post-operative survival compared to tumors with low levels of CA IX expression or no expression at all. A similar association of CA IX expression and poor prognosis has recently also been reported in non-small-cell lung cancers [12]; however, unlike gastric cancer normal lung tissue does not

express CA IX at appreciable levels. In the further analysis of the immunohistochemical sections of gastric cancers that retained CA IX expression, CA IX expression was observed primarily in cancer cells that were located at the invasion front, indicating that while a loss of CA IX expression is a frequent event in gastric cancer, those gastric tumors that retain CA IX expression exhibit increased invasiveness, which could contribute to their poor prognosis [31]. *In vivo* observations made in arriving at the instant invention are supported by the *in vitro* analysis of CA IX overexpression in AGS gastric cancer cells, described in Example 4. Upon transfection of *CA9* cDNA in such gastric cancer cells, the cell proliferation and invasive growth of the transfected cells was significantly enhanced. Thus, overexpression of CA IX in gastric cancer is also associated with enhanced cell proliferation and invasion, strengthening the finding of CA IX expression at the invasion front of gastric cancers, which also exhibit a worse prognosis.

While overexpression of CA IX has been reported in various cancers, the expression is low or even lost in most gastric cancers [15,16]. An analysis by inventors of the subject prognostic methods showed that CA IX expression was lost in the cancer cells in 26 of 57 patients, whereas in the normal stomach expression of CA IX was retained in foveolar epithelial cells and in fundic and antral glands. A previous study by Pastorekova et al. assessed the expression of CA IX in a limited number of specimens and also reported decreased CA IX expression in the gastric cancers that were studied [15]. While the loss of expression of CA IX could be interpreted as a consequence of the neoplastic changes, including dedifferentiation during gastric carcinogenesis, recent studies indicate that in fact this loss is not just an epiphenomenon but instead a critical change underlying the process of gastric carcinogenesis. That hypothesis is supported by the generation of CA IX deficient mice, in which the inactivation of the CA IX gene led to the development of gastric hyperplasia, which is associated with enhanced cellular proliferation [17]. Together with the study of inventors of the subject prognostic methods that demonstrated loss of CA IX expression in approximately half of the gastric cancers, those studies in CA IX deficient mice indicate that CA IX may function as a critical differentiation factor in the stomach that also controls cell proliferation and growth of the gastric mucosa. Indeed, the loss of CA IX expression as disclosed herein in the Western blot and PCR analyses may support the hypothesis that such a loss of CA IX

expression is critical for the development of gastric cancer and may be an early event in gastric carcinogenesis.

Nucleic Acid-Based Assays

In certain embodiments of the invention, mRNA that encodes a CA IX protein or a CA IX polypeptide or the cDNA complementary to that mRNA is detected and quantitated in a sample taken from a patient afflicted with a preneoplastic/neoplastic disease, such as gastric cancer, compared with the average of MN/*CA9* mRNA levels in comparable samples, and thereby correlated with a prognosis for a patient. Where expression of MN/*CA9* mRNA or MN/*CA9* cDNA is measured, above average *CA9* mRNA or above average *CA9* cDNA expression is indicative of a poorer prognosis. One preferred method for measuring alterations in the level of *CA9*-specific mRNA expression is Northern blotting, where the nucleic acid sequence used as a probe for detecting MN/*CA9*-specific mRNA expression is complementary to all or part of the MN/*CA9* cDNA sequence shown in Figure 1; a preparation of RNA is run on a denaturing agarose gel, and transferred to a suitable support, such as activated cellulose, nitrocellulose or glass or nylon membranes. Radiolabelled cDNA or RNA is then hybridized to the preparation, washed and analyzed by autoradiography. The samples may be stained with hematoxylin to demonstrate the histological composition of the sample, and dark field imaging with a suitable light filter illuminates the developed emulsion. Non-radioactive labels such as digoxigenin may also be used.

A second preferred method for measuring *CA9*-specific mRNA expression is detection of *CA9* mRNA expression via hybridization of a nucleic acid probe derived from MN/*CA9* cDNA sequence to RT-PCR products generated from RNA isolated from a biological sample.

Exemplary Western Blot and PCR Assays

Additionally, methods can be used in combination; for example, *CA9* mRNA and CA IX protein expression can be assessed by realtime quantitative PCR and/or by Western blotting, such as in tumor samples from patients with gastric cancer and matched samples of corresponding non-neoplastic gastric mucosa. As

described in Example 2, in all five cases in which both Western blot analysis and realtime quantitative PCR were performed in the same patient, CA IX protein levels were significantly decreased in gastric cancers compared to the matched non-neoplastic mucosa, and were associated with decreased *CA9* mRNA levels (Figure 4).

MN Gene and Protein

The terms "MN/CA IX" and "MN/*CA9*" are herein considered to be synonyms for MN. Also, the G250 antigen is considered to refer to MN protein/polypeptide [35].

Zavada et al., WO 93/18152 and/or WO 95/34650 disclose the MN cDNA sequence shown herein in FIGURES 1A-1C [SEQ ID NO: 1], the MN amino acid sequence [SEQ ID NO: 2] also shown in FIGURES 1A-1C, and the MN genomic sequence [SEQ ID NO: 3] shown herein in FIGURES 2A-2F. The MN gene is organized into 11 exons and 10 introns.

The ORF of the MN cDNA shown in FIGURE1 has the coding capacity for a 459 amino acid protein with a calculated molecular weight of 49.7 kd. The overall amino acid composition of the MN/CA IX protein is rather acidic, and predicted to have a pI of 4.3. Analysis of native MN/CA IX protein from CGL3 cells by two-dimensional electrophoresis followed by immunoblotting has shown that in agreement with computer prediction, the MN/CA IX is an acidic protein existing in several isoelectric forms with pIs ranging from 4.7 to 6.3.

The first thirty seven amino acids of the MN protein shown in FIGURES 1A-1C is the putative MN signal peptide [SEQ ID NO: 6]. The MN protein has an extracellular domain [amino acids (aa) 38-414 of FIGURES 1A-1C [SEQ ID NO: 7], a transmembrane domain [aa 415-434; SEQ ID NO: 8] and an intracellular domain [aa 435-459; SEQ ID NO: 9]. The extracellular domain contains the proteoglycan-like domain [aa 53-111: SEQ ID NO: 4] and the carbonic anhydrase (CA) domain [aa 135-391; SEQ ID NO: 5].

The CA domain is essential for induction of anchorage independence, whereas the TM anchor and IC tail are dispensable for that biological effect. The MN protein is also capable of causing plasma membrane ruffling in the transfected cells and appears to participate in their attachment to the solid support. The data evince

the involvement of MN in the regulation of cell proliferation, adhesion and intercellular communication.

MN Proteins and Polypeptides

The phrase "MN proteins and/or polypeptides" (MN proteins/polypeptides) is herein defined to mean proteins and/or polypeptides encoded by an MN gene or fragments thereof. An exemplary and preferred MN protein according to this invention has the deduced amino acid sequence shown in Figure 1. Preferred MN proteins/polypeptides are those proteins and/or polypeptides that have substantial homology with the MN protein shown in Figure 1. For example, such substantially homologous MN proteins/polypeptides are those that are reactive with the MN-specific antibodies, preferably the Mab M75 or its equivalent. The VU-M75 hybridoma that secretes the M75 Mab was deposited at the ATCC under HB 11128 on Sep. 17, 1992.

A "polypeptide" or "peptide" is a chain of amino acids covalently bound by peptide linkages and is herein considered to be composed of 50 or less amino acids. A "protein" is herein defined to be a polypeptide composed of more than 50 amino acids. The term polypeptide encompasses the terms peptide and oligopeptide.

It can be appreciated that a protein or polypeptide produced by a neoplastic cell *in vivo* could be altered in sequence from that produced by a tumor cell in cell culture or by a transformed cell. Thus, MN proteins and/or polypeptides which have varying amino acid sequences including without limitation, amino acid substitutions, extensions, deletions, truncations and combinations thereof, fall within the scope of this invention. It can also be appreciated that a protein extant within body fluids is subject to degradative processes, such as, proteolytic processes; thus, MN proteins that are significantly truncated and MN polypeptides may be found in body fluids, such as, sera. The phrase "MN antigen" is used herein to encompass MN proteins and/or polypeptides.

It will further be appreciated that the amino acid sequence of MN proteins and polypeptides can be modified by genetic techniques. One or more amino acids can be deleted or substituted. Such amino acid changes may not cause any measurable change in the biological activity of the protein or polypeptide and

result in proteins or polypeptides which are within the scope of this invention, as well as, MN muteins.

Nucleic Acid Probes

Nucleic acid probes of this invention are those comprising sequences that are complementary or substantially complementary to the MN cDNA sequence shown in Figure 1 or to other MN gene sequences, such as, the complete genomic sequence of Figures 2A-F [SEQ ID NO: 3]. The phrase "substantially complementary" is defined herein to have the meaning as it is well understood in the art and, thus, used in the context of standard hybridization conditions. The stringency of hybridization conditions can be adjusted to control the precision of complementarity. Two nucleic acids are, for example, substantially complementary to each other, if they hybridize to each other under stringent hybridization conditions.

Stringent hybridization conditions

Stringent hybridization conditions are considered herein to conform to standard hybridization conditions understood in the art to be stringent. For example, it is generally understood that stringent conditions encompass relatively low salt and/or high temperature conditions, such as provided by 0.02 M to 0.15 M NaCl at temperatures of 50° C. to 70° C. such as, 0.15 M to 0.9 M salt at temperatures ranging from 20° C. to 55° C. Less stringent conditions can be made more stringent by adding increasing amounts of formamide, which serves to destabilize hybrid duplexes as does increased temperature, such as provided by exemplary stringent hybridization conditions, such as, 0.15 M to 0.9 M NaCl in the presence of 50% formamide at 42°C. with a final wash of 0.1% SSPE and 0.1% SDS at 65° C.

Further exemplary stringent hybridization conditions are described in Sambrook et al., Molecular Cloning: A Laboratory Manual, pages 1.91 and 9.47-9.51 (Second Edition, Cold Spring Harbor Laboratory Press; Cold Spring Harbor, N.Y.; 1989); Maniatis et al., Molecular Cloning: A Laboratory Manual, pages 387-389 (Cold Spring Harbor Laboratory; Cold Spring Harbor, N.Y.; 1982); Tsuchiya et al., Oral Surgery, Oral Medicine, Oral Pathology. 71(6): 721-725 (June 1991); and in U.S. Pat. No. 5,989,838, U.S. Pat. No. 5,972,353, U.S. Pat. No. 5,981,711, and U.S. Pat. No. 6,051,226.

Only very closely related nt sequences having a homology of at least 80-90% would hybridize to each other under stringent conditions.

Antibodies

The term "antibodies" is defined herein to include not only whole antibodies but also biologically active fragments of antibodies, preferably fragments containing the antigen binding regions. Further included in the definition of antibodies are bispecific antibodies that are specific for MN protein and to another tissue-specific antigen. Humanized and fully human antibodies fall with the definition of "antibodies" herein.

Antibodies useful according to the methods of the invention may be prepared by conventional methodology and/or by genetic engineering. Antibody fragments may be genetically engineered, preferably from the variable regions of the light and/or heavy chains (V_H and V_L), including the hypervariable regions, and still more preferably from both the V_H and V_L regions. For example, the term "antibodies" as used herein includes polyclonal and monoclonal antibodies and biologically active fragments thereof including among other possibilities "univalent" antibodies [46]; Fab proteins including Fab' and $F(ab)_2$ fragments whether covalently or non-covalently aggregated; light or heavy chains alone, preferably variable heavy and light chain regions (V_H and V_L regions), and more preferably including the hypervariable regions [otherwise known as the complementarity determining regions (CDRs) of the V_H and V_L regions]; F_c proteins; "hybrid" antibodies capable of binding more than one antigen; constant-variable region chimeras; "composite" immunoglobulins with heavy and light chains of different origins; "altered" antibodies with improved specificity and other characteristics as prepared by standard recombinant techniques and also oligonucleotide-directed mutagenesis techniques [47].

The antibodies useful according to this invention to identify CA IX proteins/polypeptides can be labeled in any conventional manner, for example, with enzymes such as horseradish peroxidase (HRP), fluorescent compounds, or with radioactive isotopes such as, ^{125}I, among other labels. A preferred label, according to this invention is ^{125}I, and a preferred method of labeling the antibodies is by using chloramine-T [48].

Representative monoclonal antibodies useful according to this invention include Mabs M75, MN9, MN12, MN7 and V/10 described in earlier Zavada et al. patents and patent applications. [US Pat. No. 6,297,041; US Pat. No. 6,204,370; US Pat. No. 6,093,548; US Pat. No. 6,051,226; US Pat. No. 6,004,535; US Pat. No. 5,989,838; US Pat. No. 5,981,711; US Pat. No. 5,972,353; US Pat. No. 5,955,075; US Pat. No.5,387,676; US Application Nos: 20030049828 and 20020137910; and International Publication No. WO 03/100029]. Monoclonal antibodies useful according to this invention serve to identify MN proteins/polypeptides in various laboratory prognostic tests, for example, in clinical samples. For example, monoclonal antibody M75 (Mab M75) is produced by mouse lymphocytic hybridoma VU-M75, which was deposited under ATCC designation HB 11128 on September 17, 1992 at the American Tissue Type Culture Collection [ATCC]. The production of hybridoma VU-M75 is described in Zavada et al., International Publication No. WO 93/18152. Mab M75 recognizes both the nonglycosylated GST-MN fusion protein and native CA IX protein as expressed in CGL3 cells equally well. The M75 Mab recognizes both native and denatured forms of the CA IX protein [56].

General texts describing additional molecular biological techniques useful herein, including the preparation of antibodies include Berger and Kimmel, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol. 152, Academic Press, Inc., Sambrook et al., Molecular Cloning: A Laboratory Manual, (Second Edition, Cold Spring Harbor Laboratory Press; Cold Spring Harbor, N.Y.; 1989) Vol. 1-3; Current Protocols in Molecular Biology, F. M. Ausabel et al. [Eds.], Current Protocols, a joint venture between Green Publishing Associates, Inc. and John Wiley & Sons, Inc. (supplemented through 2000), Harlow et al., Monoclonal Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1988), Paul [Ed.]; Fundamental Immunology,Lippincott Williams & Wilkins (1998), and Harlow et al., Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1998).

MATERIALS AND METHODS

Subjects. Tumorous and corresponding non-tumorous paraffin embedded tissue specimens from 59 patients (20 female, 39 male, age range 41-84

years) were retrieved from the archive of the Institute of Pathology of the University of Magdeburg for immunohistochemical analyses. 27 patients had suffered from diffuse type and 32 from intestinal type gastric cancer, according to the Lauren classification [18]. For molecular analyses gastric cancer and corresponding non-lesional tissue were obtained immediately after surgery from 18 patients with gastric cancer (2 female, 16 male, age range 43-82 years). Tissue samples were snap-frozen in liquid nitrogen and stored at –80°C and further processed as described below, or fixed in 10% neutralized formalin and embedded in paraffin for histological processing. The study was approved by the Human Subjects Committee of the University of Magdeburg, Germany.

Cell lines. The gastric cancer cell lines MKN45, MKN28, AGS, N87 and the Hela cells were obtained from Riken Cell Bank (Tsukuba, Japan) and the American Type Culture Collection (ATCC, Rockville, MD). All cell lines, except AGS and Hela cells, were maintained in RPMI medium (Gibco BRL, Rockville, MD, USA) with 10% fetal bovine serum. The AGS cell line was kept in F-12K medium with 10% fetal bovine serum and the Hela cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum.

Transient transfection assay. AGS gastric cancer cells were seeded at a density of 2 x 10^5 cells/60 mm dish. The cells were transfected 24 hrs later with a pSG5C vector (5µg) containing the human *CA9* cDNA (1.5 kb, KpnI/SacI site)[3], or an empty pCMVβ vector (control B) or were incubated with the Transfectam reagent alone (control A) (Promega, Mannheim) according to the manufacturer's recommendations with the optimal volume/weight ratio of Transfectam Reagent/DNA of 2µl/µg DNA. Protein expression was confirmed after 24hrs, 48hrs and 72hrs by Western blot analysis (not shown).

Treatment of cells with 5-aza-dC. Cells were seeded at a density of 1 x 10^6 cells/60 mm dish. Twenty-four hours later, the cells were treated with 5 µM 5-aza-dC (Sigma Chemical Co., Deisenhofen, Germany). The same concentration of DMSO was also used as a control for nonspecific solvent effect on cells. Total cellular protein was isolated 3 days after addition of 5-aza-dC as previously described [19].

Cell proliferation assay. AGS cells were grown in media supplemented with 10 % fetal calf serum (Gibco Invitrogen) and 50 µg/ml rifobacin. Parental AGS cells, AGS cells transfected with the pCMVβ vector and CA IX transfected AGS cells were seeded in 96 well plates at a density of 30 x 104 cells/200 µl/well. After 40 hrs of culture at 37°C, 5 % (v/v) CO_2, cells were pulsed for an additional 8 hrs with $_3$H-methyl-thymidine (0.2 µCi/well), and harvested onto glass fibre membranes. The incorporated radioactivity was measured by scintillation counting. In each case DNA synthesis was assessed 6 times in parallel and repeated once, resulting in a total of 12 experiments per cell line [20].

In vitro invasion assay. Cellular invasion of AGS cells was evaluated in 24-well Transwell chambers (Costar, Bodenheim, Germany) as described previously [21]. The upper and lower culture compartments were separated by polycarbonate filters with 8 µm pore size. Prior to invasion assays, the polycarbonate filter was coated with 100 ng matrigel matrix. For invasion assays, 3 x 10^4 cells per well were incubated on the reconstituted basement membrane for 72 hrs. Cells passing the filters and attaching to the lower sites of matrigel-coated membranes were harvested using trypsin/EDTA; the cell number was quantified in a Coulter Counter ZII (Coulter Immunotech, Marseille, France). The number of migrating cells was calculated from controls grown under identical culture conditions for 72 hrs in 24 well plates. All experiments were performed in triplicate.

Real-time quantitative analysis of CA9 mRNA levels. Tissue specimens were homogenized with an ultrasound homogenizer (Ultra-Turrax T25 basic, IKA, Staufen, Germany). Total RNA (1 µg) was reverse transcribed at 37°C for 1 hr in a final volume of 20 µl reverse transcription buffer (50 mM Tris-HCl pH 8.3, 7 mM $MgCl_2$ and 40 mM KCl and 10 mM DTT) containing 100 U MMLV reverse transcriptase, Rnase H Minus, Point Mutant (Promega, Mannheim, Germany), 16 U RNase inhibitors (Promega), 200 pmol random primer (Promega) and 0.5 mM dNTPs (Biomol Feinchemikalien, Hamburg, Germany). Briefly, PCR primers were designed to amplify a 240 bp cDNA fragment of the CA IX gene (sense 5'-AGGAGGATCTGCCCAGTGA -3' [SEQ ID NO: 10]; antisense 5'-GCCAATGACTCTGGTCATC -3') [SEQ ID NO: 11][4]. The expression level of CA IX was determined by using the LightCycler technique (Roche Diagnostics GmbH, Mannheim, Germany) as previously described [20].

Immunohistochemistry. Deparaffinized serial sections were cut at 3 µm for immunohistochemistry and placed on Superfrost Plus glass slides. Immunostaining was performed with a monoclonal antibody M75 directed against CA IX [15]. For immunostaining, sections were deparaffinized in xylene and rehydrated in an alcohol series. Anti-CA IX (dilution 1:10) was administered for 1 hr at 37 °C in a moist chamber, followed by incubation with biotinylated anti-mouse IgG/anti-rabbit IgG (1:200; Vector Laboratories; distributed by Camon, Wiesbaden, Germany) and ABC alkaline phosphatase reagent, each for 30 min at room temperature. Between steps the sections were washed in Tris buffered saline. Immunoreactions were visualized with the avidin biotin complex method applying a Vectastain ABC alkaline phosphatase kit (distributed by Camon, Wiesbaden, Germany). Neufuchsin served as chromogen. All specimens were counterstained with hematoxylin. Primary antibodies were omitted for negative controls.

Evaluation of immunohistochemical results. A numerical scoring system with two categories was used to assess the observed expression of CA IX in tumor cells and gastric epithelium. Category A documented the number of immunoreactive cells as 0 (no immunoreactive cells), 1 (<10%), 2 (11 to 50%), and 3 (>50%). A positive case was defined as having a Category A value of 1. Category B documented the intensity of the immunostaining as 0 (no immunostaining), 1 (weak), 2 (moderate), and 3 (strong). Finally, the values for Category A and B were added to give the "immunoreactivity score" (IRS), which could range from 0 to 6. Note that the method of calculating the IRS does not allow the individual categories to add up to an IRS of 1.

Western blot analysis. Human gastric tissues and cell lines were lysed in a buffer containing 1 mM EDTA, 50 mM β-glycerophosphate, 2 mM sodium orthovanadate, 1% Triton-100, 10% glycerol, 1 mM DTT and protease inhibitors (10 mg/ml benzamidine, 2 mg/ml antipain, and 1 mg/ml leupeptin). After separation, proteins were electroblotted onto polyvinylidene difluoride membranes (Bio-Rad). The membrane was incubated with 1:200 anti-CA IX M75 antibody for 1 hr at room temperature, as previously described [20]. Membrane-bound secondary antibodies were detected by enhanced chemiluminescence following the instructions of the manufacturer. To ensure equal loading amounts, the blots were stripped in 200 mmol/L glycine, 1% Tween-20, 0.1% SDS, pH 2.2, for 2 hrs at room temperature and

rehybridized using a monoclonal anti-β-actin antibody (dilution 1:2000; clone AC-74; Sigma) [19].

Statistical analysis. The number of proliferating/invading cells and the expression of CA IX was analysed using student's t test. The survival curve was plotted using the Kaplan-Meier method, and comparison of survival times was performed with the log-rank test. A p value < 0.05 was taken as the level of significance.

The following examples are for purposes of illustration only and are not meant to limit the invention in any way.

Example 1

Localization of CA IX expression in gastric cancer cells

The distribution and expression pattern of CA IX was investigated by immunohistochemistry. Tissue sections were stained with anti-CA IX antibody M75. CA IX was found in the non-neoplastic gastric mucosa, intestinal metaplasia and significantly less often in gastric cancer. Occasionally the immunostaining was heterogeneous: for example, poorly differentiated (G3) intestinal type gastric cancer exhibited no immunostaining of the tumor cells in the mucosa, and intense staining in a subset of the tumor cells infiltrating the muscularis propria (Hematoxylin counterstain).

Overall, CA IX was found in non-neoplastic gastric mucosa of every patient studied. It was confined to foveolar epithelial cells, fundic and antral glands. Intestinal metaplasia was observed in 15 (26.3%) patients, and CA IX was expressed at the brush border of the intestinal metaplasia in 10 patients (66.7%). CA IX was expressed in gastric cancer cells of 31 (54.0%) patients. No differences were found between intestinal and diffuse type of gastric cancer. CA IX was present in the tumor cells of 14 (51.9%) patients with diffuse type gastric cancer, in 12 (57.1%) patients with moderately differentiated intestinal type gastric cancer and 5 (55.6%) patients with poorly differentiated intestinal type gastric cancer. The mean total immunoreactivity score for CA IX was calculated to be 1.95±1.98 for gastric cancer compared with 5.66±0.78 in the foveolar epithelium ($p<0.001$). Again no

differences were found between diffuse or intestinal type gastric cancers. Table 1 summarizes the total immunoreactivity scores for CA IX.

Table 1

Expression of CA IX in gastric cancer as shown by immunohistochemistry

Characteristics	Moderately differentiated intestinal type	Poorly differentiated intestinal type	Diffuse type
	n=21	n=9	n=27
Age (years±SD)	68.3±10.0	60.0±11.2	62.0±11.0
Gender (m/f)	15/6	7/2	15/12
[a]IRS of cancer cells	1.67±1.73	2.05±1.99	1.96±2.10
IRS of foveolar epithelium	5.50±0.84	5.88±0.33	5.52±0.98
[b]P-value	<0.001	<0.001	<0.001

[a]IRS denotes immunoreactivity score; [b] The P-value refers to the differences between the IRS of cancer cells and foveolar epithelium.

Example 2

Quantitative analysis of CA IX expression in gastric cancers

CA9 mRNA and CA IX protein expression were then assessed by realtime quantitative PCR and Western blotting. Tumor samples were obtained from 18 patients with gastric cancer and matched corresponding non-neoplastic gastric mucosa was also available from those patients. *CA9* mRNA levels in cancer and non-cancer tissues were assessed in 10 patients, whereas Western blot analysis was performed in 12 cases. In 5 cases, both Western blot analysis and realtime quantitative PCR was performed in the same patient, allowing a direct comparison of the expression levels of CA IX protein and *CA9* mRNA in gastric cancer and non-neoplastic gastric mucosa. Overall the levels of CA IX protein and *CA9* mRNA were significantly decreased in gastric cancers compared to the matched non-neoplastic mucosa (p=0.04). The direct comparison of 5 cases in which both CA IX protein and

CA9 mRNA levels were assessed, revealed that in all cases reduced protein levels were associated with decreased *CA9* mRNA levels (Figure 4).

Example 3

Prognostic significance of CA IX expression in gastric cancer

Survival data were obtained from 23 patients with gastric cancer undergoing gastric cancer resection. According to the immunohistochemical score as outlined above two groups of patients were classified as group A with low CA IX expression (IRS $\leq$3) versus group B with high CA IX expression in the cancer cells (IRS > 3). Post-operative survival time for patients with high CA IX expression was significantly shorter than in patients without or low CA IX expression (p=0.0281) (Figure 5). Interestingly, expression of CA IX was very prominent at the site of infiltration of the muscularis propria, indicating that despite the overall loss of CA IX expression in gastric cancer, the sustained or re-expression of CA IX at the invasion front may contribute to the overall poor survival in patients with increased CA IX expression.

Example 4

CA IX transfection induces invasion and proliferation of AGS cells

CA9 mRNA and CA IX protein-levels were investigated in AGS, N87 and MKN28 gastric cancer cells by realtime PCR and Western blotting. HeLa cells served as positive control. *CA9* mRNA and CA IX protein were found in N87 and MKN28 cells, albeit at significantly lower levels compared to HeLa cells (Figure 4). *CA9* mRNA and CA IX protein were undetectable in AGS cells, which were then chosen for transfection of *CA9* cDNA in order to assess the biological changes associated with CA IX expression. AGS cells were transfected with full-length *CA9* cDNA, with an empty expression vector (control B) or were treated only with the transfectam reagent without DNA transfer (control A). The expression of CA IX in transfected cells was confirmed by Western blotting (not shown). The invasive capability of transfected AGS cells was assessed using 24-well Transwell chambers. The expression of CA IX in AGS cells resulted in a significant increase of migrating

cells compared with controls, i.e. incubation of parental AGS cells with transfectam only or AGS cells transfected with empty vector (controls A and B) (Figure 6). Furthermore, transfected AGS cells showed a significant increase in cell proliferation compared with the two control groups (Figure 6).

Example 5

Biological effects of restoration of CA IX expression in gastric cancer cells by inhibition of methylation

The levels of *CA9* mRNA were also analyzed in N87, MKN28, MKN45 and AGS cells after treatment with 5′-aza-deoxycytidine, a demethylating agent. Treatment with 5′-aza-deoxycytidine increased *CA9* mRNA levels more than 5 fold in N87, MKN45 and AGS cells, indicating that the expression of CA IX is, at least in part, regulated by methylation. No effect was observed in MNK28 cells (Figure 7). The *in vitro* matrigel invasion assay was used to assess the invasive potential of 5′-aza-deoxycytidine treated AGS cells compared to untreated AGS cells. Untreated AGS cells exhibited no striking difference in invasiveness as compared to DMSO treated AGS cells (control). In contrast, 2.9% of 5′-aza-deoxycytidine treated AGS cells passed the reconstituted matrigel matrix, while only 1.05% of untreated and 1.04% of DMSO treated AGS cells were detectable on the lower side of the filters ($p<0.01$) (Figure 7).

DISCUSSION

Recent studies in renal cancer indicate that CA IX expression is, at least in part, regulated by methylation of the *CA9* gene promoter and that hypomethylation of CpG at –74 bp and –6 bp sites in the *CA9* promoter region is associated with increased CA IX expression in human renal cancer cell lines [32, 33]. Since as shown herein reduced or lost expression of CA IX was observed in a large number of gastric cancers and gastric cancer cell lines, 4 well-established gastric cancer cell lines were treated with 5'-aza-deoxycytidine, a demethylating agent, in order to analyze whether inhibition of methylation might lead to the restoration of CA IX expression. As described in Example 5 above, all cell lines, except for MKN28 cells, exhibited increased *CA9* mRNA levels in the realtime PCR analysis after treatment with 5'-aza-deoxycytidine, indicating that the expression of

CA IX in gastric cancer cell lines is, at least in part, regulated by methylation of CpG sites [see Figure 7]. Since reexpression of CA IX was observed in those cells after treating the cells with 5'-aza-deoxycytidine, it appeared fruitful to assess, whether the restoration of CA IX expression would also alter the biological characteristics of the cells. Therefore, the cells were also analyzed in an invasion assay that allowed for the evaluation of changes in invasiveness of cells with and without treatment. While no changes were observed in the invasiveness of the AGS cells, which were kept in media or DMSO added to the media, AGS cells incubated with 5'-aza-deoxycytidine exhibited a significant, almost 3 fold increased level of invasiveness, indicating that the restoration of CA IX in those cells is associated with enhanced invasion.

Based on the data reported herein, it can be assumed that the loss of CA IX is an early event in gastric cancer, that may be associated with an increased promoter methylation. Later in the process of gastric cancer progression CA IX expression is induced at the invasion front of the cancer cells, which gives those cells an additional growth advantage by enhancing their proliferation and invasive growth. Inasmuch as HIF-1α is induced by intratumoral hypoxia, which in turn induces CA IX expression [24, 25], it is assumable that the reexpression of CA IX at the invasion front of gastric cancers may result from the activation of the O_2-regulated subunit of HIF-1 leading to increased HIF-1α expression at the invasion front of gastric cancers, which has already been reported in colon and other cancers [34]. In summary, while the frequent loss of CA IX expression observed in gastric cancer may be an early event, the overexpression of CA IX at the invasion front of a subset of gastric cancers may lead to invasive growth and thereby contributes to the growth and progression of gastric cancer malignancy. The inventors then conclude that preneoplastic/neoplastic diseases having similar CA IX expression patterns as that of gastric cancer would also be subject to the prognostic methods disclosed herein.

Budapest Treaty Deposits

The materials listed below were deposited with the American Type Culture Collection (ATCC) now at 10810 University Blvd., Manassus, Virginia 20110-2209 (USA). The deposits were made under the provisions of the Budapest Treaty on the International Recognition of Deposited Microorganisms for the Purposes of

Patent Procedure and Regulations thereunder (Budapest Treaty). Maintenance of a viable culture is assured for thirty years from the date of deposit. The hybridomas and plasmids will be made available by the ATCC under the terms of the Budapest Treaty, and subject to an agreement between the Applicants and the ATCC which assures unrestricted availability of the deposited hybridomas and plasmids to the public upon the granting of patent from the instant application. Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any Government in accordance with its patent laws.

Hybridoma	Deposit Date	ATCC #
VU-M75	September 17, 1992	HB 11128
MN 12.2.2	June 9, 1994	HB 11647

Plasmid	Deposit Date	ATCC #
A4a	June 6, 1995	97199
XE1	June 6, 1995	97200
XE3	June 6, 1995	97198

Similarly, the hybridoma cell line V/10-VU which produces the V/10 monoclonal antibodies was deposited on February 19, 2003 under the Budapest Treaty at the International Depository Authority (IDA) of the Belgian Coordinated Collections of Microorganisms (BCCM) at the Laboratorium voor Moleculaire Biologie-Plasmidencollectie (LMBP) at the Universeit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium [BCCM/LMBP] under the Accession No. LMBP 6009CB.

The description of the foregoing embodiments of the invention have been presented for purposes of illustration and description. They are not intended to be exhaustive or to limit the invention to the precise form disclosed, and obviously many modifications and variations are possible in light of the above teachings. The embodiments were chosen and described in order to explain the principles of the invention and its practical application to enable thereby others skilled in the art to

utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated.

All references cited herein are hereby incorporated by reference.